ANNUAL PROGRESS REPORT

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Prepared by K. S. Pilcher for period January 1 to December 31, 1953.

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TITLE OF PROJECT: A study of the effect of certain arginine analogs and other metabolite analogs on the multiplication of typical animal viruses.

OBJECTIVES: The objectives of this project may be stated as follows:

1. To complete the study of the inhibitory properties of isopropyl biguanide, benzoyl guanylurea, and canavanine for the Lee influenza virus. This implies the use of experimental infections in the intact chick embryo, in mice, and in tissue cultures. Other viruses, including influenza A, mumps, and equine encephalomyelitis, will also be included in the study.

2. To investigate a new and interesting lead which has recently turned up in our work from the observation that cyanuric acid also has the ability to inhibit the Lee influenza virus in the chick embryo. This observation seems to merit thorough study.

3. To continue the synthetic program aimed at the synthesis of canavanine and various derivatives and analogs of the latter. Encouraging progress along this line has recently been made. Compounds resulting from this program are to be compared with canavanine from the standpoint of antiviral properties.

4. To continue to examine by preliminary tests for antiviral activity selected compounds which may become available and whose structure suggests the possibility of biological antagonism.

PROGRESS:

A. Examination of Compounds for Influence on Virus Multiplication:

1. Materials and Methods:

The only new method employed during the period of this report has been a method for cultivation of Lee influenza virus in tissue culture. The technique used has been essentially that described by Tamm, Folkers and Horsfall (6). Chorioallantoic membranes from 10-12 day chick embryos were washed with nutrient fluid and cut into two portions, each having a wet weight between 130 and 170 mgm. Each portion was suspended in 2 ml. of nutrient fluid in a 25 x 150 mm. Pyrex tube fitted with a rubber stopper. After inoculation with virus, culture tubes were incubated at 35°C. on a reciprocating shaker having a rate of 90 strokes per minutes and a stroke length of 8 cm.

The nutrient fluid was prepared as follows: modified glucosol was made up by the Fulton and Armitage formula (7). It contained NaCl, 8 gm.; $CaCl_2$, 0.2 gm.; $MgCl_2.6H_2O$, 0.5 gm.; glucose, 1.0 gm.; glass distilled H_2O to 1000 ml. M/15 phosphate buffer of pH 7.28 was made by dissolving Na_2HPO_4, 7.105 gm., KH_2PO_4, 2.269 gm., and phenol red, 40 mgm., in glass distilled water and making up to 1000 ml. The glucosol and buffer were autoclaved separately and mixed in equal volumes as required. This mixture constituted the nutrient fluid. Penicillin and streptomycin were added to give a concentration of 10 units and 40 micrograms per ml. respectively.

Compounds to be tested in tissue cultures were first dissolved in freshly mixed nutrient fluid. If pH adjustment was required, it was accomplished by the addition of N/10 HCl or NaOH. The solution was then sterilized by filtration through a fritted glass filter.

The virus employed for inoculation of tissue cultures was either freshly harvested allantoic fluid pooled from embryos giving the highest hemagglutinin titers, or else similar material which had been stored at -20°C. for no longer than a week.

Hemagglutinin titration of virus in tissue cultures was carried out in the same manner as for allantoic fluids. This was described in the report for May 1 to July 1, 1952.

2. Results:

Evaluation of Compounds for Influence on the Development of Lee Influenza Virus in the Chick Embryo.

(1) Results of preliminary tests of compounds for inhibitory activity.

In the previous report covering the period July 1 to December 31, 1952, most of the work described the inhibitory effect of canavanine on the growth of the Lee influenza virus in the chick embryo and experiments designed to investigate the nature of this inhibition. One of the primary problems involved in this study was the source of supply

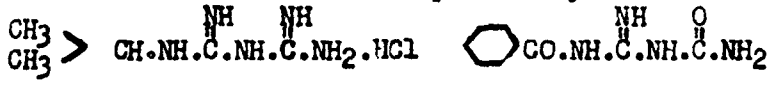
6. Tamm, I., Folkers, K., and Horsfall, F. L. Yale J. Biol. & Med. 24:559 (1952).

of canavanine itself as this amino acid must be extracted from Jack bean meal by a laborious process. In view of the fact that the rate of progress of this work was limited by the supply it was decided to devote this period to a study of other compounds of potential interest while a stock pile of canavanine was being built up.

An interesting group of compounds bearing a structural relationship to arginine became available to us during this period. Samples were originally obtained through the kindness of Dr. I. F. Halverstadt of the Chemical Research Department of Cutter Laboratories. These compounds included guanylurea derivatives and biguanides. They were synthesized originally by the Product Development Department of Merck and Company, and further quantities have been obtained from the latter group. Additional compounds were also obtained through the courtesy of Dr. B. E. Christensen, Professor of Organic Chemistry, Oregon State College.

The results of the preliminary tests on this group of compounds, and a few others examined at about the same time, are presented in Table A. These tests were only intended to indicate whether a compound might have an influence on the development of the virus and whether it was worthy of further investigation. It will be noted that two of the compounds in this table appear to have had a rather striking inhibitory effect on the growth of the virus as judged by the hemagglutinin titer of the pooled allantoic fluids from the treated eggs. These are isopropyl biguanide hydrochloride and benzoyl guanylurea. As judged by the results of this test with a very small number of eggs, both of these compounds prevented the development of sufficient virus to be detectable in the pooled fluids by hemagglutination. These compounds obviously were worthy of further investigation.

The structure of these two compounds may be indicated as follows:



isopropyl biguanide hydrochloride

benzoyl guanylurea

Although they differ somewhat, it is obvious that they have in common the guanidino grouping which is of course found in arginine.

It will be noted also that three other compounds, i.e. cyclohexyl guanylurea, guanylurea phosphate and disodium versene also reduced the virus titers of the pooled allantoic fluids below the level of the controls. Statistically, significance cannot be attached to any of these differences, but the data suggest some slight activity by these compounds. The only other compound which produced a result which might be considered different from the control is naphthoxyacetic acid. The hemagglutinin level here was three times that of the control eggs, suggesting stimulation of virus development. However, we have attempted to confirm this result and have been unsuccessful. Apparently some conditions prevailed in this particular test which we have not been able to duplicate.

7. Fulton, F. and Armitage, P. J. Hyg. 49:247 (1951).

TABLE A

Results of Further Preliminary Tests with Various Organic Compounds for Possible Inhibitory Activity Against Lee Influenza Virus in the Chick Embryo

Compound Tested	Dose per Egg	Dose of Virus per Egg	Fraction of Eggs Showing Virus Hemagglutinin	Hemagglutinin Titer of Pooled Fluids from All Eggs in Group*
Phenyl biguanide hydrochloride	6 mg.	50 ID_{50}	8/8	120
Isopropyl biguanide hydrochloride	12 mg.	"	0/3	0#
Cyclohexyl guanylurea	2 mg.	"	6/7	60
Benzoyl guanylurea	25 mg.	"	1/6	0
Dodecyl guanylurea	0.125 mg.	"	5/5	120
Guanylurea phosphate	25 mg.	"	7/8	60
Disodium versene	3 mg.	"	7/11	60
2,6-diaminopurine	5 mg.	"	8/8	160
Orotic acid	50 mg.	"	7/8	240
2,4-Dichlorophenoxyacetic acid	25 mg.	"	5/6	120
Naphthoxyacetic acid	10 mg.	"	8/8	480
None; Controls receiving 1 ml. carboxymethyl cellulose	--	"	7/7	160
None; Controls receiving virus only	--	"	7/8	160

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.
Eggs were incubated at 35°C. approximately 44 hours before virus titrations were made.

From the foregoing results it was concluded that an investigation of the effects of isopropyl biguanide hydrochloride and benzoyl guanylurea on development of the virus was justified.

During the period of this report other compounds have been examined by this preliminary test for possible inhibition of the Lee influenza virus. Some of these compounds are analogs of arginine, which were synthesized for this purpose, such as argininic acid, and nitroarginine. Others are structurally related to canavanine, which we have found inhibitory to the Lee virus. Desaminocanavanine, carboxymethoxy guanidine, and ethoxy guanidine fall in this category. The latter two were synthesized, while desaminocanavanine was prepared from canavanine. The results obtained with these compounds are shown in Table B. Some of the other compounds in this table were selected because they have some structural similarity to guanylurea or biguanides, such as biuret and nitrourea. In addition, some heterocyclic nitrogen compounds were included, such as cyanuric acid and citrazinic acid.

It will be noted in Table B that cyanuric acid appeared to inhibit the virus, and that 4-(B, γ-dihydroxy-propylamino)-7-chloroquinazoline also appeared inhibitory to a lesser degree. Confirmatory experiments with both of these compounds have been completed, and cyanuric acid again showed pronounced inhibition, with less than 10 hemagglutinin units per ml. in the pooled fluids from treated eggs, compared to 320 units per ml. in control fluids. The chloroquinazoline derivative did not give the same result when tested the second time, and the reason for the discrepancy has not been investigated.

It does seem evident, however, that cyanuric acid is quite effectively inhibitory for this virus and that this effect deserves further investigation.

Several pyrimidine compounds were tested, and these together with a few miscellaneous compounds appear in Table C. The data suggest that uracil-4-acetic acid, and cyclohexane dione have inhibitory properties. More extensive tests with these compounds have verified this observation, although it has not been possible as yet to investigate either of them further. Because of the relationship of pyrimidines to nucleic acid metabolism it would appear that uracil-4-acetic acid at least should be explored further.

(2) Further investigation of the inhibition of the Lee influenza virus by isopropyl biguanide* and benzoyl guanylurea.

The nature of the inhibition of the Lee virus by the above two compounds has been investigated fairly extensively. Most of the work, however, has been carried out with isopropyl biguanide because of the rather limited supply of benzoyl guanylurea, which

* Wherever the term isopropyl biguanide is used in this report it is understood that the compound is in the form of the hydrochloride.

TABLE B

Results of Further Preliminary Tests with Guanidino, Heterocyclic Nitrogen and Related Compounds for Possible Inhibitory Activity Against Lee Influenza Virus in the Chick Embryo

Compound Tested	Dose per Egg	Fraction of Eggs Showing Virus Hemagglutinin Experiment Number						Hemagglutinin Titer* of Pooled Fluids from All Eggs in Group Experiment Number					
		4	5	6	7	8	9	4	5	6	7	8	9
Nitrourea	25.0 mg.	4/7	5/6					240	240				
Biuret	25.0 mg.		6/6						240				
Dithiobiuret	2.0 mg.		5/6						240				
Cyanuric acid	12.5 mg.		0/6						0				
Arginine nitrate	15.0 mg.			3/4						40			
Arginimic acid	15.0 mg.			6/6						160			
Carboxymethoxy guanidine	25.0 mg.			4/4						120			
4-(B,γ-dihydroxy-propylamino) -7-chloroquinazoline	50.0 mg.			1/5						<20			
Nitroarginine	50.0 mg.				5/5						160		
Cincophene	15.0 mg.				7/7						320		
Ethoxyguanidine	10.0 mg.					2/2						240	
Desaminocanavanine	20.0 mg.					3/4						320	
Citrazinic acid	20.0 mg.					8/8						640	
Pipecolinic acid	50.0 mg.						4/6						320
Control; 1.0 ml. of 1% carboxymethyl cellulose		6/7	6/6		6/6			320	240	120	240		
Control; 0.5 ml. of sterile distilled H_2O		6/7	2/3	4/4	5/6	8/8	7/8	320	120	120	160	320	160

* Reciprocals of highest fluid dilutions giving complete hemagglutination. Eggs were incubated at 35°C. approximately 44 hours before virus titrations were made.

TABLE C

Results of Further Preliminary Tests with Pyrimidine Derivatives and Miscellaneous Compounds for Possible Inhibitory Activity Against Lee Influenza Virus in the Chick Embryo

Compound Tested	Dose per Egg	Fraction of Eggs Showing Virus Hemagglutinin — Experiment Number						Hemagglutinin Titer* of Pooled Fluids from All Eggs in Group — Experiment Number					
		4	5	6	7	8	9	4	5	6	7	8	9
2-Thiouracil	50 mg.	2/5						120					
Uracil-4-acetic acid	50 mg.			1/4						10			
DL-Dihydroxythymine	6.0 mg.				6/8						240		
2-methyl-mercapto-pyrimidone	25.0 mg.						6/7						120
Hexamethylene bis thiuronium bromide	5.0 mg.				2/4						240		
Cyclohexane dione	2.5 mg.					2/9						40	
Hexamethylene bis (1,3 diisopropyl-thiuronium bromide)	2.5 mg.						1/3						160
Sodium copper chlorophyll	5.0 mg.						5/6						160
Control; 1 ml. of 1% carboxymethyl cellulose		6/7	6/6		6/6			320	240	120	240		
Control; 0.5 ml. of sterile distilled H_2O		6/7	2/3	4/4	5/6	8/8	7/8	320	120	120	160	320	160

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
Eggs were incubated at 35°C. approximately 44 hours before virus titrations were made.

could not be obtained in additional quantity.

To begin with, the meager data from the preliminary test was supplemented by further studies in which the fluids from individual eggs were titrated for virus hemagglutinin. The results of a typical experiment employing isopropyl biguanide are presented in Table 1. In this experiment as in all others in this report unless indicated otherwise, injections of both virus and compound were made into the allantoic sac. It is evident from these results that the Lee influenza virus in the chick embryo is markedly inhibited by 10.0 mg. of isopropyl biguanide for at least 44 hours after inoculation, as judged by the hemagglutinin titers.

The results of a similar experiment in which the dose of isopropyl biguanide was injected in the yolk sac of the chick embryo while the virus was injected into the allantoic sac are given in Table 2. From these data it seems apparent that it is not necessary to have direct contact between the compound and the virus in the allantoic sac in order to demonstrate inhibition of the virus. These results suggest that the compound may be absorbed into the circulation of the embryo and in some manner interfere with the growth of the virus in infected cells.

The results of more detailed observation of the effect of benzoyl guanylurea on the Lee influenza virus are shown in Table 3. From inspection of this table it also seems quite obvious that this compound too has a very marked inhibitory effect upon the development of the virus as judged by the hemagglutinin titer. In comparing results obtained with the two compounds it should be noted that a dose of 10.0 mg. of isopropyl biguanide dissolved in a volume of 0.2 ml. of distilled water was regularly employed. This had been found to be about the maximum tolerated dose. However, benzoyl guanylurea has a much lower solubility and in the case of this compound it was necessary to employ a suspension made up to a concentration of 50 mg. per ml. in a suspending medium of one percent low viscosity carboxy-methyl cellulose. The maximum tolerated dose of the compound had been found to be about 25.0 mg. Thus it is hard to compare the activity of the two compounds weight for weight because of the large difference in solubility.

Experiments were next carried out to determine the minimum effective dose of isopropyl biguanide which could cause inhibition of the virus. Results of these experiments appear in Table 4. It will be noted that the inhibitory effect drops off rather rapidly with decreasing dosage. 10.0 mg. per egg inhibited hemagglutinin development completely. The inhibition produced by a dose of 5.0 mg. per egg was still marked and in the case of 2.5 mg. per egg was still significant. A slight effect may have resulted from the use of 1.0 mg. per egg, but the difference between the result obtained at this dose level and the control group is not significant.

Similar experiments were carried out with benzoyl guanylurea and these are given in Table 5. The effect of the decreased dosage

is even more apparent in the case of this compound. 25.0 mg. per egg caused almost complete suppression of hemagglutinin whereas 12.5 mg. per egg did not cause any significant reduction in the hemagglutinin titer.

An effort was next made to determine over how long a period the inhibitory effect of isopropyl biguanide could be detected. All of the previous observations had been made after a period of approximately forty-four hours of incubation at 35°C. Accordingly, observations were made on both treated and control eggs on both the third and fourth day of incubation. These results appear in Table 6. It will be noted that after sixty-eight hours of incubation treated eggs still showed a level of virus hemagglutinin which was only about twenty per cent of that found in the untreated controls. After ninety-two hours of incubation the titer of the pooled fluids from the treated embryos was about half as great as the comparable control group. Although this difference has not been tested for statistical significance it seems doubtful that with the relatively small number of eggs involved, such a difference could be demonstrated. However, it is quite possible that further expansion of the number of observations would reveal such a difference. In any event it seems apparent that the inhibitory effect of a single dose of 10 mg. of isopropyl biguanide, which is very marked after forty-four hours of incubation, is still marked after sixty-eight hours and is at best very slight after ninety-two hours of incubation.

TABLE 1

Inhibitory Effect of Isopropyl Biguanide on Lee Influenza Virus in the Chick Embryo as Measured by the Hemagglutination Reaction

Egg No.	Hemagglutinin Titers of Fluids from Eggs Receiving 50 ID_{50} of Virus Only	Hemagglutinin Titers of Fluids from Eggs Receiving 10 mg. Isopropyl Biguanide an Hour Before 50 ID_{50} of Virus
1	320*	20*
2	320	0#
3	320	0
4	240	0
5	240	0
6	240	0
7	160	0
8	80	0
9	40	0
10	0	0
Pool of Fluids from all Eggs in Group	160	0

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.
Eggs were incubated at 35°C. approximately 44 hours before virus titrations were made.

TABLE 2

Inhibitory Effect of Isopropyl Biguanide Injected in the Yolk Sac on Lee Influenza Virus Injected in the Allantoic Sac of Chick Embryos

Egg No.	Hemagglutinin Titers of Fluids from Eggs Receiving 50 ID_{50} of Virus only in Allantoic Sac	Hemagglutinin Titers of Fluids from Eggs Receiving 10 mg. Isopropyl Biguanide in the Yolk Sac an Hour Before 50 ID_{50} of Virus in Allantoic Sac.
1	640*	0#
2	640	0
3	480	0
4	320	0
5	320	0
6	320	0
7	320	0
8	320	-
9	160	-
10	120	-
Pool of Fluids from all Eggs in Groups	480	0

* Reciprocals of highest fluid dilutions giving complete hemagglutinations.
\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.
Eggs were incubated at 35°C. approximately 44 hours before virus titrations were made.

TABLE 3

Inhibitory Effect of Benzoyl Guanylurea on Lee Influenza Virus in the Chick Embryo as Measured by the Hemagglutination Reaction

Egg No.	Hemagglutinin Titers of Fluids from Eggs receiving 50 ID_{50} of Virus Only	Hemagglutinin Titers of Fluids from Eggs Receiving 25 mg. Benzoyl Guanylurea an Hour Before 50 ID_{50} of Virus
1	640	40*
2	240	20
3	240	0#
4	240	0
5	240	0
6	160	0
7	160	0
8	120	0
9	120	-
10	80	-
Pool of Fluids from all Eggs in Group	320	0

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.
Eggs were incubated at 35°C. approximately 44 hours before virus titrations were made.

TABLE 4

Effect of Dosage of Isopropyl Biguanide on the Inhibition of Lee Influenza Virus in the Chick Embryo

Dose of Isopropyl Biguanide per Egg	Fraction of Eggs Showing Virus Hemagglutinin	Number of Eggs Showing Titers of (0)	(20-80)	(120-320)	(480 & above)	Hemagglutinin Titers of Pooled Fluids from all Eggs in Group*
10.0 mg.	1/10	9	1	0	0#	0
5.0 mg.	4/10	6	3	1	0	30
2.5 mg.	3/7	4	1	1	1	120
1.0 mg.	9/10	1	3	5	1	240
0.5 mg.	10/10	0	2	6	2	320
none	9/10	1	2	5	2	320

All eggs received 50 ID_{50} of virus approximately an hour after the injection of the compound. They were then incubated at 35°C. approximately 44 hours before virus titrations were made.

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.

TABLE 5

Effect of Dosage of Benzoyl Guanylurea on the Inhibition of Lee Influenza Virus in the Chick Embryo

Dose of Benzoyl Guanylurea per Egg	Fraction of Eggs showing Virus Hemagglutinin	Number of Eggs Showing Titers of (0)	(20-80)	(120-320)	(480 & above)	Hemagglutinin Titers of Pooled Fluids from all Eggs in Group*
25.0 mg.	2/8	6	2	0	0#	0
12.5 mg.	10/10	0	0	9	1	120
6.3 mg.	9/10	1	2	6	1	80
none	10/10	0	1	8	1	160

All eggs received 50 ID_{50} of virus approximately an hour after the injection of the compound. They were then incubated at 35°C. approximately 44 hours before virus titrations were made.

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
\# Fluid dilutions below 1:20 were not treated, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.

TABLE 6

Duration of Inhibiting Effect of Isopropyl Biguanide on Lee Influenza Virus in the Chick Embryo

	Fraction of Eggs Showing Virus Hemagglutinin	Number of Eggs Showing Titers of (0)	(20-80)	(120-320)	(480 & above)	Hemagglutinin Titers of Pooled Fluids from All Eggs in Group*
Eggs incubated 68 hours at 35°C. after receiving 10 ID_{50} of Lee Virus	9/10	1	0#	5	4	320
Eggs incubated 68 hours at 35°C. after receiving 10 mg. IBG+ and 10 ID_{50} of Lee Virus an hour later	9/9	0	8	1	0	60
Eggs incubated 92 hours at 35°C. after receiving 10 ID_{50} of Lee Virus	10/10	0	0	5	5	480
Eggs incubated 92 hours at 35°C. after receiving 10 mg. IBG+ and 10 ID_{50} of Lee Virus an hour later	8/8	0	1	7	0	240

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.
\+ IBG - isopropyl biguanide

The influence of the dose of virus upon the inhibitory effect of isopropyl biguanide has been investigated. In these experiments all treated eggs received 10 mg. of the compound in the allantoic sac about an hour before the virus was inoculated by the same route. After 44 hours incubation the allantoic fluids from all eggs were titrated individually for virus hemagglutinin. The results of typical experiments bearing on this point are presented in Table 7. It is obvious that the dose of virus has a definite influence upon the final result. When the inoculum was small complete suppression of the virus hemagglutinin was observed. When the dose of virus was increased, the inhibitory effect of the compound diminished. With 1000 ID_{50} of virus the effect was still significant, but with 10,000 ID_{50} only a slight, questionable difference was found between control and treated eggs. Attempts to use more than 10,000 ID_{50} were unsatisfactory because of irregularity of infection and lower hemagglutinin titers, resulting presumably from auto interference effects.

A study has also been made of the influence of the time interval between injection of the virus and injections of isopropyl biguanide on the inhibition of the virus in the chick embryo. The data bearing on this point are presented in Table 8. It will be noted that there was no apparent difference in the end result if the compound was injected an hour before the virus or two hours after the virus. In both cases the development of virus hemagglutinin was almost completely suppressed. When the compound was administered twenty-four hours after the virus, the inhibitory effect was still marked and the virus hemagglutinin in treated eggs was only about ten per cent of that found in controls. When the injection of the compound was delayed until thirty-six hours after infection with the virus a slight inhibitory effect was still found. When this time interval, however, was increased to forty-four hours no significant effect was demonstrable. It is interesting to note that the inhibition is still so striking, even when injection of the compound is delayed for twenty-four hours after infection. This observation suggests that the mechanism of inhibition is not concerned primarily with interference with adsorption of the virus to the susceptible cells, but is more likely related to some step in the formation of the virus itself or of its release from the cells.

A certain amount of similar data has been obtained with benzoyl guanylurea. This is much more limited because of the limited quantity of this compound which was available. These results appear in Table 9. It will be noted that here also essentially identical results were obtained if the compound was given either an hour before or two hours after the virus. In both cases the development of virus hemagglutinin was markedly suppressed. When the compound was given as late as twenty-four hours after the virus, however, only a slight inhibitory effect was noted. Thus the results with this compound are much less striking when treatment is delayed for twenty-four hours than is the case with isopropyl biguanide.

It is, of course, of interest to know whether these compounds have any direct inactivating effect on the Lee influenza virus in

TABLE 7

Influence of Virus Dose Level on the Inhibitory Effect of Isopropyl Biguanide on Lee Influenza Virus in the Chick Embryo

Exp. No.	Dose of Virus Injected	Dose of Isopropyl Biguanide	Fraction of Eggs Showing Virus Hemagglutinin	Number of Eggs Showing Titers of				Hemagglutinin Titers of Pooled Fluids from All Eggs in Group *
				0	(20-80)	(120-320)	480 & above	
1	7 ID_{50}	10 mg.	0/10	10	0	0	0#	0
"	7 ID_{50}	none	8/10	2	2	5	1	160
"	70 ID_{50}	10 mg.	5/10	5	5	0	0	30
"	70 ID_{50}	none	10/10	0	0	8	2	320
2	1000 ID_{50}	10 mg.	7/10	3	1	5	1	160
"	1000 ID_{50}	none	10/10	0	0	4	6	480
"	10,000 ID_{50}	10 mg.	9/10	1	1	8	0	160
"	10,000 ID_{50}	none	9/10	1	0	7	2	320

All eggs that were to receive isopropyl biguanide were injected about an hour before the injection of the indicated amount of virus. They were then incubated at 35°C. approximately 44 hours before virus titrations were made.

In Exp. No. 1, lyophilized virus was used; in Exp. No. 2, fresh virus.

* Reciprocals of highest fluid dilutions giving complete hemagglutination.

\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.

TABLE 8

Influence of Time Interval Between Infection and Injection of Isopropyl Biguanide on the Inhibition of Lee Influenza in the Chick Embryo

10 mg. Isopropyl Biguanide Injected	Fraction of Eggs Showing Virus Hemagglutinin	Number of Eggs Showing Titers of				Hemagglutinin Titers of Pooled Fluids from all Eggs in Group *
		0	(20-80)	(120-320)	(480 & above)	
1 hour before virus	3/10	7	3	0	0#	0
2 hours after virus	1/10	9	1	0	0	0
24 hours after virus	9/18	9	7	2	0	30-40+
36 hours after virus	14/16	2	5	9	0	120+
44 hours after virus	10/10	0	2	4	4	320
Controls receiving virus only	20/20	0	1	9	10	320-480

All eggs received 50 ID_{50} of virus. 10 mg. Isopropyl biguanide was administered at the indicated times. Eggs were incubated at 35°C. for approximately 48 hours before virus titrations were made.

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.
\+ Data represents combined results of 2 experiments.

TABLE 9

Influence of Time Interval Between Infection and Injection of Benzoyl Guanylurea on the Inhibition of Lee Influenza in the Chick Embryo

25 mg. Benzoyl Guanylurea Injected	Fraction of Eggs Showing Virus Hemagglutinin	Number of Eggs Showing Titers of				Hemagglutinin Titers of Pooled Fluids from all Eggs in Group *
		0	(20-80)	(120-320)	(480 & above)	
1 hour before virus	4/10	6	4	0	0#	20
2 hours after virus	1/8	7	1	0	0	0
24 hours after virus	8/8	0	2	6	0	120
Controls receiving virus only	9/10	1	1	8	0	240

All eggs received 50 ID_{50} of virus. 25 mg. of benzoyl guanylurea was administered at the indicated times. This was suspended in a 1% aqueous solution of carboxymethylcellulose to give a concentration of 25 mg. per ml. Control eggs received 1 ml. of the suspending agent only an hour before the virus. Eggs were incubated at 35°C. for approximately 48 hours before virus titrations were made.

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.

vitro. Experiments bearing on this point are recorded in Table 10. Isopropyl biguanide has been the only compound investigated and a concentration of 2 mg. per ml. has been employed, as this is estimated to be the maximum concentration produced in the allantoic fluid of the chick embryo when a dose of 10 mg. is administered. Periods of contact between the virus and the compound of two hours and twenty hours have been tested at temperatures of 10°C. and 35°C. It will be noted from the table that in no case was there any effect upon the hemagglutinin titer of the virus. In the case of the virus infectivity, however, it is apparent that in the presence of the compound after twenty hours at 35°C. this property of the virus deteriorated more rapidly than in a control preparation containing only the virus. The pH of the two preparations was approximately the same so that the difference noted could not be ascribed to a pH effect. At 10°C., after a twenty hour period of exposure a preparation containing isopropyl biguanide was slightly less active than the control but the difference can probably not be considered significant. Similarly after a period of two hours at 35°C. the preparation containing isopropyl biguanide showed a slightly lower infectivity than the control preparation.

These observations have been extended by additional experiments, with closely similar results. No effect of the compound on the hemagglutinating activity of the virus was noted within 24 hours at 35°C., nor upon virus infectivity within 24 hours at 10°C. or 2 hours at 35°C. However, within 24 hours at 35°C. the presence of the compound again accelerated the loss of virus infectivity. It is difficult to judge the importance of this relatively mild in vitro effect in relation to the in vivo inhibition of the virus. It hardly seems adequate to account for all aspects of the latter, but cannot be ruled out as at least a contributing factor.

The effect of isopropyl biguanide upon the development of the Lee influenza virus has been measured by means of infectivity titrations as well as by hemagglutinin titrations. This method has not been used routinely because it is less precise and more laborious, but the inhibitory effect can be demonstrated by either method. Infectivity titers of virus from eggs treated with isopropyl biguanide have usually been at least one log unit lower than the titers found in untreated eggs.

Since the last report further experiments have been carried out to determine the possible effect of isopropyl biguanide on the adsorption of the virus by chorio-allantoic membrane tissue in vitro. In these experiments freshly harvested allantoic fluid virus was mixed with fresh minced chorio-allantoic membrane tissue in the proportion of 1 gram of tissue per ml. of fluid, both with and without the addition of isopropyl biguanide at a concentration of 2 mg. per ml. This was judged to be the maximum concentration reached in the fluids of chick embryos receiving 10 mg. of the compound. It had no influence on the pH of the virus fluid at this concentration. These mixtures, together with controls containing virus only, and virus plus isopropyl biguanide, without tissue, were incubated at 35°C. for 30 minutes, then centrifuged to remove the tissue, and the supernatants titrated for virus hemagglutinin.

The original virus content of the fluid was approximately $10^{7.4}$ EID_{50} per ml. In some experiments the virus fluid was diluted 2 fold, 5 fold, or 10 fold with normal allantoic fluid to give lower virus concentrations for adsorption.

It was found that the proportion of virus adsorbed by the tissue in the absence of the compound varied from 50% to 67% when undiluted virus was used. With virus diluted 1:2 or 1:5 about 75% was adsorbed, and with virus diluted 1:10, more than 90%. In the presence of the compound, the proportion of virus adsorbed did not differ significantly from the above values, although in some cases the data suggested that in the mixtures containing the compound there may have been slightly more unadsorbed virus. Hence it would appear that if there is any interference with adsorption, it must be too slight to be readily detected by the methods employed.

(3) Effect of isopropyl biguanide on the growth of other viruses in the chick embryo.

The influence of isopropyl biguanide on the development of PR8 influenza virus, mumps virus and western equine encephalomyelitis virus in the chick embryo has been studied.

In the case of the PR8 virus, the experimental method employed was identical with that used for the Lee virus. A dose of 50 EID_{50} was administered in the allantoic sac about an hour after the injection of 10 mg. of the compound by the same route. After 44 hours incubation at 35°C. the allantoic fluids were individually titrated for virus hemagglutinin. The results of a typical experiment with this virus are shown in Table 11. It will be noted that the degree of inhibition noted with this virus was far less than in the case of the Lee virus although it was still of sufficient magnitude to be statistically significant. It is interesting to note that this degree of specificity exists with respect to the inhibitory action of isopropyl biguanide. It would appear to be a reflection of some fundamental difference in these two types of influenza virus and may be related to the difference in their biochemical composition which has been reported by Knight (8).

The method employed in testing the effect of this compound against mumps virus was essentially similar to that employed for the influenza virus. The chief difference was in the time of administration of the compound, which, in the case of the mumps virus, was 24 hours after inoculation of the virus itself. It was found that if the compound was given at the same time as the virus or if the dose was divided in two parts which were administered at the time of inoculation of virus and 48 hours later, no inhibitory effect on the growth of the virus was noted. However, if a single dose of 10 mg. of the compound was given 24 hours after the virus, some slight to moderate inhibition was evidenced. The results of an experiment with mumps virus appear in Table 12. In this case the mean virus titer in control embryos was about 7 fold greater than that found in treated embryos. In some experiments the degree of inhibition noted was considerably less. There seems to be no doubt,

8. Knight, C. A., J. Exp. Med. 86:125 (1947).

TABLE 10

In Vitro Effect of Isopropyl Biguanide on Lee Influenza Virus

Exp. No.	Time and Temperature of Incubation		Hemagglutinin* Titer	No. of EID_{50}+ of Virus per ml.
1	None	Virus only before incubation	160	$10^{7.15}$
"	2 hours at 10°C.	Virus only	160	$10^{7.60}$
"	2 hours at 10°C.	Virus + IBG#; 2 mg. per ml.	160	$10^{7.40}$
"	2 hours at 35°C.	Virus only	160	$10^{7.74}$
"	2 hours at 35°C.	Virus + IBG; 2 mg. per ml.	160	$10^{7.17}$
2	None	Virus only before incubation	240	$10^{6.61}$
"	20 hours at 10°C.	Virus only	240	$10^{7.50}$
"	20 hours at 10°C.	Virus + IBG; 2 mg. per ml.	240	$10^{7.00}$
"	20 hours at 35°C.	Virus only	320	$10^{3.64}$
"	20 hours at 35°C.	Virus + IBG; 2 mg. per ml.	320	$10^{2.00}$

* Reciprocals of highest dilutions of fluid giving complete hemagglutination.
\# IBG = isopropyl biguanide.
\+ EID_{50} = quantity of virus needed to infect 50% of inoculated chick embryos.

TABLE II

Effect of Isopropyl Biguanide on PR8 Influenza Virus in the Chick Embryo as Measured by the Hemagglutination Reaction

Egg No.	Hemagglutinin Titers of Fluids from Eggs Receiving 50 ID_{50} of Virus Only	Hemagglutinin Titers of Fluids from Eggs Receiving 10 mg. Isopropyl Biguanide an Hour Before 50 ID_{50} of Virus
1	480	320
2	320	120
3	240	80
4	240	80
5	240	80
6	160	60
7	160	60
8	160	60
9	120	40
10	120	0
11	120	
Pool of fluids from all eggs in group	320	120
Geometric mean	194.4	66.5

Figures in table are reciprocals of highest dilutions of virus fluids giving complete agglutination.

Fluid dilutions below 1:20 were not tested, and fluids giving a completely negative reaction at this dilution are arbitrarily assigned a value of 0.

When the above results were tested by the "t" test for comparison of means, using the logs of the hemagglutinin titers, a "t" value of 3.8 was obtained. For 19 degrees of freedom this is equivalent to a P value of <0.01, indicating the above differences would occur by chance less than once in 100 trials.

TABLE 12

Effect of Isopropyl Biguanide on Mumps Virus in the Chick Embryo as Measured by the Hemagglutination Reaction

Egg No.	Hemagglutinin Titers of Fluids from Eggs Receiving 50 ID_{50} of Virus Only	Hemagglutinin Titers of Fluids from Eggs Receiving 10 mg. Isopropyl Biguanide 24 Hours After 50 ID_{50} of Mumps Virus
1	160	30
2	80	20
3	60	20
4	60	20
5	60	10
6	40	10
7	40	10
8	40	0
9	30	0
10	0	0
Pool of fluids from all eggs in group	60	10
Geometric mean	47.0	6.9

Figures in table are reciprocals of highest dilutions of virus fluids giving complete agglutination.

Fluid dilutions below 1:20 were not tested. Fluids giving 3+ agglutination at this dilution were considered to be completely active (4+) at 1:10 and were assigned a value of 10. Those completely negative at 1:20 were given a value of 0.

however, that a definite though relatively slight degree of inhibition of the mumps virus is produced by isopropyl biguanide.

In the case of western equine encephalomyelitis virus an entirely different test method was essential. Groups of ten-day chick embryos were inoculated with about 10 EID_{50} of the virus in the allantoic sac. Similar groups of embryos which had received 10 mg. of isopropyl biguanide by the same route about an hour previously were also inoculated with the virus. All embryos were then candled at 8-hour intervals to determine the time of death. If the compound had interfered significantly with the development of the virus, death of the treated embryos should have been delayed in comparison with the untreated controls. This was not found to be the case, however, as the majority of the embryos in both groups had succumbed within 48 hours and no differences could be noted between the two groups. From this it would appear that isopropyl biguanide does not interfere with the development of this neurotropic virus.

(4) Effect of canavanine and isopropyl biguanide on the Lee influenza virus in tissue culture.

It was considered to be desirable to investigate the effects of canavanine and isopropyl biguanide on the development of Lee influenza virus in tissue culture. This was based on the assumption that under these circumstances the inhibitory effect of these two compounds, which has already been demonstrated in the intact chick embryo, might be magnified in tissue culture and might be demonstrable with considerably lower concentrations than are required in the chick embryo. Under these circumstances there might be a better opportunity for demonstrating a reversal of the inhibitory effect by certain natural metabolites.

The tissue culture technique employed has been previously described in the section on methods. The minimum concentration of canavanine sulfate required to inhibit development of the virus in tissue culture was determined. The results of experiments bearing on this point are presented in Tables 13 and 14. It will be noted there that the concentration of 0.2 mg. per ml. completely suppressed development of virus hemagglutinin, .1 mg. per ml. produced marked inhibition, and .05 mg. per ml. resulted in slight inhibition. These observations seem to indicate that the effective concentration in tissue culture is considerably less than that required in the allantoic fluids of the intact chick embryo. It will be recalled that to produce the maximum degree of inhibition in the latter case a dose of about 20 mg. per embryo is required, which is equivalent to a concentration in the allantoic fluid of the order of 2.0 mg. per ml. This is approximately ten times the concentration found to be effective in tissue culture. It now remains to determine whether inhibition of the virus by canavanine in tissue culture may be reversed by the presence of certain known metabolites.

TABLE 13

Inhibition of Lee Influenza Virus in Tissue Culture
by Canavanine Sulfate

Culture Number	Virus Hemagglutinin Present in Tissue Cultures Containing the Following Concentrations of Canavanine Sulfate			
	0.5 mg. per ml.	0.2 mg. per ml.	0.1 mg. per ml.	No canavanine; controls
1	0	0	120	320
2	0	0	40	240
3	0	0	20	240
4	0	0	10	240
5	0	0	0	160
6	0	0	0	160
Geometric mean	0	0	9.9	220.0

1. All figures in table represent reciprocals of highest dilutions of tissue culture fluids giving complete hemagglutination. Dilutions below 1:20 were not tested. If a culture was entirely negative at 1:20, it was assigned a value of 0.

2. Virus titrations were made after 44 hours incubation at 35°C. on a reciprocating shaker.

3. The virus inoculum was 0.1 ml. of a 1:10 dilution of allantoic fluid virus freshly harvested from chick embryos.

TABLE 14

Inhibition of Lee Influenza Virus in Tissue Culture by Canavanine Sulfate

Culture Number	Virus Hemagglutinin Present in Tissue Cultures Containing the Following Concentrations of Canavanine Sulfate			
	0.05 mg. per ml.	0.02 mg. per ml.	0.01 mg. per ml.	No canavanine; controls
1	160	120	240	240
2	120	120	160	240
3	120	120	160	160
4	80	80	160	160
5	60	80	160	120
6	40	80	160	120
Geometric mean	87.3	113.1	171.2	166.4

Figures in table are reciprocals of highest dilutions of virus fluids giving complete agglutination.

Fluid dilutions below 1:20 were not tested, and fluids giving a completely negative reaction at this dilution are arbitrarily assigned a value of 0.

Most of the tissue culture work carried on during the period of this report has been confined to a study of isopropyl biguanide. It was first necessary to determine the minimum effective concentration of this compound required for inhibition of the virus and also the influence of the dose of inoculated virus employed in the cultures upon the inhibition. A summary of some of the more pertinent results obtained in this study is presented in Table 15. It will be noted that a concentration of the compound as low as .1 mg. per ml. may produce marked inhibition of virus development in the cultures. This is much less than the concentration required for a similar degree of inhibition in the intact chick embryo. In the latter case a concentration of the order of 1.0 mg. per ml. of allantoic fluid is needed. It will also be noted in Table 15 that a given concentration of the compound becomes less effective as the dose of inoculating virus is increased. This observation is entirely analogous to what has been found in the case of infection of the intact chick embryo. On the basis of these results it was decided that further tissue culture experiments designed to study the effect of known metabolites on the inhibition of the virus should be carried out with a concentration of .1 mg. isopropyl biguanide per ml. and with a virus inoculum of .1 ml. of a 1:10 dilution of freshly harvested allantoic fluid virus. It would, of course, have been more satisfactory to prepare a pool of virus to be used as inoculum in these experiments and to preserve this pool by storage at -60°C., but this method could not be employed because no refrigerator capable of maintaining this temperature is available.

It was felt to be necessary to determine whether isopropyl biguanide in the concentrations to be employed might produce sufficient permanent damage to the tissue fragments in the cultures so that they would be incapable of supporting a virus growth. For this purpose experiments were set up in which the tissues were exposed to various concentrations of the compound in the usual nutrient fluids for a period of 36-48 hours. At this time culture fluids were removed and replaced with fresh fluid containing none of the compound. These cultures were then inoculated with virus, reincubated and titrated for virus content after an additional 48-hour period. It was found that with concentrations of isopropyl biguanide greater than .2 mg. per ml. it was not possible to demonstrate virus development when the cultures were inoculated after replacement of the fluid. However, with either .1 or .2 mg. of the compound per ml. exposure of the tissues to the compound did not render them incapable of supporting virus multiplication. This information also indicated the desirability of employing a concentration of .1 mg. per ml. in further studies.

A number of known metabolites have so far been tested for the ability to prevent or interfere with inhibition of the virus by isopropyl biguanide. These include arginine, lysine, adenine, p-aminobenzoic acid, folic acid, and desoxyribonucleic acid (sodium salt). Although these experiments have been in most cases technically satisfactory, it has not been possible, thus far at least, to show any clear-cut evidence that any of these compounds is capable of interfering with inhibition of the virus by isopropyl biguanide. The result of a typical experiment of this type is

TABLE 15

Relationship of Dosage of Isopropyl Biguanide and of Virus Inoculum to the Inhibition of Lee Influenza Virus in Tissue Culture

Culture Number	Virus Hemagglutinin Present in Tissue Cultures Containing the Following Concentrations of Isopropyl Biguanide with the Indicated Virus Inoculum								
	0.1 mg. IBG per ml. with			0.2 mg. IBG per ml. with			0.5 mg. IBG per ml. with		
	$10^{7.2613}$ EID50 of Virus per ml.	$10^{6.2613}$ EID50 of Virus per ml.	$10^{5.2613}$ EID50 of Virus per ml.	$10^{7.2613}$ EID50 of Virus per ml.	$10^{6.2613}$ EID50 of Virus per ml.	$10^{5.2613}$ EID50 of Virus per ml.	$10^{7.2613}$ EID50 of Virus per ml.	$10^{6.2613}$ EID50 of Virus per ml.	$10^{5.2613}$ EID50 of Virus per ml.
1	80	20	0	80	10	0	20	0	0
2	80	2-10	0	30	2-10	0	10	0	0
3	80	0	0	30	2-10	0	2-10	0	0
4	40	0	0	30	0	0	2-10	0	0
5	30	0	0	20	0	0	2-10	0	0
6	30	0	0	10	0	0	0	0	0
Control Cultures Receiving Virus Only in the Indicated Dosage									
1	640			480			960		
2	640			320			480		
3		480			320			320	
4		160			160			240	
5			40			60			60
6			20			60			10

1. Reciprocals of highest fluid dilutions giving complete hemagglutination.
2. Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0. Fluids giving slight or partial agglutination at this dilution were assigned the range value of 2-10.
3. Virus inoculum was from a frozen stock of allantoic fluid virus held at -20°C. for no more than 7 days. It was titrated for infectivity in chick embryos. The volume of inoculum in each case was 0.1 ml.
4. Virus titrations were made after 44 hours incubation at 35°C. on a reciprocating shaker.

presented in Table 16, where p-aminobenzoic acid was examined for any possible reversing effect. This compound was tested because it has been reported to be capable of reversing the antimalarial effect of chlorguanide, an antimalarial compound which is closely related structurally to isopropyl biguanide. These results, however, give no indication that the antiviral effect of the latter compound can be influenced by p-aminobenzoic acid. Experiments of this type are being continued in the hope that it may be possible to shed some further light on the mechanism of inhibition of the Lee virus by isopropyl biguanide.

(5) Tests with isopropyl biguanide in experimental infection of mice with the Lee influenza virus.

Thus far two experiments have been performed to determine whether isopropyl biguanide can influence the course of infection with the Lee influenza virus in mice. In the first experiment groups of mice were given intraperitoneal injections of 6 mg. of isopropyl biguanide in aqueous solution. On the first day of the experiment all treated mice, together with an equal number of controls, were inoculated intranasally with .05 ml. of allantoic fluid virus. One group of mice received a 10^{-4} dilution of the allantoic fluid while the second group received a 10^{-2} dilution. The experiment was continued for 10 days, during which time the treated mice received daily injections of the compound. At the end of this period all survivors were autopsied and examined for extent of pulmonary lesions. No significant differences could be noted between treated and control groups either with respect to the number of survivors or the extent of pulmonary lesions.

In the second experiment an attempt was made to provide a more constant blood level of isopropyl biguanide by preparing the compound as a suspension in sesame oil containing aluminum monostearate. Treated animals were injected daily with .05 ml. of this suspension containing 15 mg. intramuscularly. In this case half the mice were inoculated with a 10^{-2} dilution of the virus; the other half with a 10^{-3} dilution. At the end of 10 days the groups were again compared on the basis of survivors and extent of pulmonary lesions. Again, however, no significant differences could be noted between treated and control groups.

It is planned to carry out one further experiment with this compound in mice in which the virus inoculum used will be from a strain of the Lee virus that has been thoroughly adapted to mice. The purpose of this will be to minimize the dose of virus which must be used in order to produce a fatal infection in the mice. At the present time, however, there is no evidence that isopropyl biguanide can influence the course of infection with the Lee influenza virus in mice.

TABLE 16

Results of Tests with p-Aminobenzoic Acid and Isopropyl Biguanide in Tissue Cultures of Lee Influenza Virus

Exp. No.	Culture Number	Hemagglutinin Titers in Tissue Cultures Containing 0.1 mg. Isopropyl Biguanide per ml. plus: PABA 4 mg/ml	PABA 1 mg/ml	PABA 0.2 mg/ml	PABA 0.04 mg/ml	no PABA	Hemagglutinin Titers in Control Cultures; no Isopropyl Biguanide or PABA
1	1	20	0	0	20	0	160
	2	20	0	0	0	0	80
	3	2-10	0	0	0	0	80
	4	0	0	0	0	0	60
	5	0	0	0	0	0	40
	6	0	0	0	0	0	30
		PABA 5 mg/ml				no PABA	Control Cultures
2	1	2				2	60
	2	2				2	60
	3	2				2	60
	4	2				2	60
	5	2				2	60
	6	2				2	40
	7	2				0	40
	8	0				0	30
	9	0				0	30
	10	0				0	30
	11	0				0	30
							20

All figures in table represent reciprocals of highest dilutions giving complete hemagglutination.
All cultures were tested after 44 hours incubation on shaker at 35°C.

B. Preparation of Compounds for Antiviral Testing.

The work on an improved method for isolation of canavanine has been continued. Using slight refinements in the procedure some 35 g. have been prepared. Several more small scale attempts to isolate canavanine by the use of ion exchange resins have been made. Paper chromatography indicates that arginine and histidine follow canavanine through the columns. Colorimetric analysis indicates that canavanine is present in the acid eluate but attempts at isolation have been unsuccessful. It is thought that decomposition may occur on the ion exchange resin as has been reported to occur on an adsorption column (Archibald[1]).

To check this possibility pure canavanine was run through the resins. According to the colorimetric method the IRC - 50 resin buffered at pH 4.7 retained 92% of the canavanine; when buffered at pH 7.0 it retained 27%; and the IRA-400 resin retained 69%. However, the flavianate prepared from these acid eluates did not correspond to canavanine flavianate; this may be due to the formation of a eutectic mixture with ammonium flavianate or to flavianates of decomposition products of canavanine. This is being investigated further.

5-aminopyrimidinedione - 2,4; 5-nitropyrimidinedione - 2,4; 5-α,α-dichloroacetamidopyrimidinedione - 2,4; 2-thio-4-oxypyrimidine, 2-methylmercapto-4-hydroxypyrimidine, cyanuric acid and biuret have been prepared by procedures described in the literature. The colorimetric determination for canavanine (Archibald[1]) has been extended to alcoholic solutions. Canaline has been made enzymatically and desaminocanavanine prepared by the method of Kitagawa[2].

Plans for the immediate future include the isolation of more canavanine and the preparation of certain of its derivatives.

C. Attempted Synthesis of Compounds Related to Canavanine.

In 1929 Kitagawa[3] discovered a hitherto unknown basic amino acid in Jack bean meal. It was named Canavanine and was shown to have the constitutional formula, $NH_2C(=NH)-NHOCH_2CH_2CH(NH_2)COOH$. It was the first derivative of hydroxy guanidine ever found in nature. Upon hydrolysis of canavanine, urea and Canaline, $NH_2OCH_2CH_2CH(NH_2)COOH$, are obtained as products. Kitagawa was able to synthesise canaline from 4-hydroxy-2-amino butanoic acid and

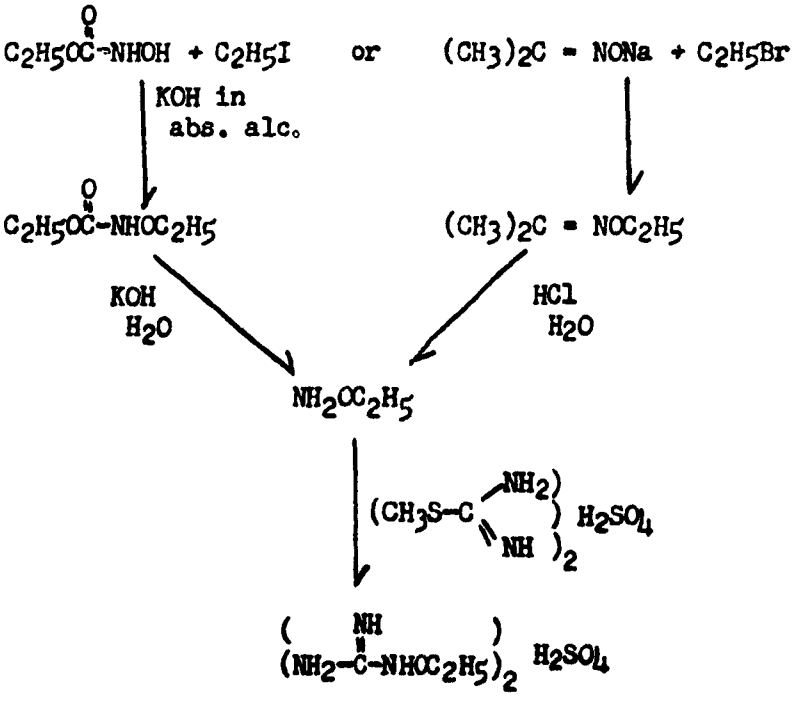
1. Archibald, R. M.; J. Biol. Chem. 165, 169 (1946).
2. Kitagawa, M.; J. Biochem. (Japan), 25, 23 (1941).
3. Kitagawa, M.; J. Biochem. (Japan), II, 205.

subsequently obtain canavanine by condensation of canaline with methylisourea. However, Kitagawa made his starting material from canaline itself and his synthesis cannot be considered as complete.

Borek and Clarke[4] in 1938 attempted the complete synthesis of canaline but abandoned it after unexpected difficulties set in. They did, however, synthesize three previously unknown derivatives of hydroxy guanidine. These were methoxy guanidine, carboxymethoxy guanidine, and 3-carboxypropoxy guanidine. Nothing more was reported with regard to the synthesis of derivatives of hydroxy guanidine until 1947 when Fuller and King[5] reported their preparation of a number of alkoxy and alkylenedioxy guanidine compounds.

The efforts very briefly summarized above constitute the only work done on the synthetic problems of these compounds.

Two derivatives of hydroxy guanidine have recently been prepared in the current synthetic program. The compounds prepared were carboxymethoxy guanidine, $NH_2-C(=NH)-NHOCH_2COOH$, and ethoxy guanidine sulfate, $(NH_2-C(=NH)-NHOC_2H_5)_2\ H_2SO_4$. Ethoxy guanidine sulfate is a new compound not previously reported in the literature. It was made by the following sequence of reactions:



4. Borek and Clarke; J. Biol. Chem., 125, 479-494.
5. Fuller and King; J. Chem. Soc., 963-969 (1947).

Carboxymethoxy guanidine was mentioned previously as being synthesized by Borek and Clarke.

At the present time the synthesis of 3-methyl-butoxy guanidine, $NH_2C(=NH)-NHOCH_2CH_2CH(CH_3)_2$, and 2-amino-3-guanidoxy propanoic acid, $NH_2C(=NH)-NHOCH_2CH(NH_2)COOH$, is being attempted. Neither of these compounds has been reported in the literature before. It will be noted that 2-amino-3-guanidoxy propanoic acid is the next lower homologue of canavanine. The tentative scheme of synthesis is as follows:

$(CH_3)_2C = NONa + BrCH_2CH(OE+)_2$

$(CH_3)_2C = NOCH_2CH(OE+)_2$

$\xrightarrow{H_2O,\ H^+}$ $(CH_3)_2C = NOCH_2CHO$

$\xrightarrow{NH_4CN}$ $(CH_3)_2C = NOCH_2CH(NH_2)-CN$

$(CH_3)_2C = NOCH_2CH(NH_2)COOH$

$NH_2C(=NH)-NHOCH_2CH(NH_2)COOH$

The preparation of 3-methylbutoxy guanidine is being done by the same scheme of synthesis as was followed for ethoxyguanidine sulfate. Work on both of these compounds is being pursued at the present time and the first reaction step for each of them has been worked out.

SUMMARY OF RESULTS SINCE BEGINNING OF PROJECT:

A. Prior to Present Report Period.

1. Two groups of compounds have been examined for any possible influence on the development of the mumps virus in the chick embryo. One of these groups may be considered to be analogs of the amino acid arginine. The other group is composed of purine and pyrimidine compounds. Of the eight compounds examined altogether none was found to have any influence upon the growth of the mumps virus.

2. The same two groups of compounds with the addition of two or three others have been tested against the Lee influenza virus in the chick embryo. Of the five compounds in the purine and pyrimidine group, none was found to have any influence on the growth of the virus. However, of seven compounds in the arginine analog group, two were found to influence the development of the Lee influenza virus. Canavanine sulphate, an amino acid isolated from jack bean meal showed a definite inhibitory effect while DL-citrulline exerted a slight stimulating effect.

3. Studies on the minimum dose of canavanine required to produce inhibition of the Lee virus indicate that 20 milligrams per egg, approximately the maximum tolerated dose, produces the most marked degree of inhibition, but some inhibition can still be observed at a level of 5 milligrams per egg.

4. The dose level of virus may be varied between 10 and 1,000 ID_{50} without obscuring the inhibitory effect of canavanine. Higher dosage levels of virus remain to be tested.

5. The inhibitory effect of canavanine on the Lee influenza virus is most marked when the compound is administered to the embryo about an hour before the virus inoculum. However, slight degrees of inhibition may still be noted when the compound is given as late as 24 hours after the virus.

6. The mechanism of action of canavanine in producing inhibition of the Lee virus is being studied. At this point it does not appear that the compound has any direct inactivating effect on the virus.

7. The administration of DL-citrulline to chick embryos in a dosage of 50 milligrams about an hour before inoculation of Lee influenza virus stimulates the development of the virus to the extent that the mean titer of hemagglutinin in treated eggs is about double that found in controls.

8. L-canavanine sulfate has been isolated from jack bean meal by the method of Horowitz and Fling (9). Yields averaged 7-8 grams per 900 grams of meal.

9. Several other compounds have been synthesized for testing, including DL-citrulline, N-benzoyl arginine, and DL-2,4-diaminobutanoic acid.

B. During Present Report Period.

1. Of a number of additional compounds examined five have been found to have a definite inhibitory effect on the development of the Lee influenza virus in the chick embryo. These are isopropyl biguanide hydrochloride, benzoyl guanylurea, cyanuric acid, uracil-4-acetic acid, and cyclohexane dione. The first two compounds contain the guanidino group and in this respect

9. Horowitz, N. H. and Fling, Marguerite. Private communication.

bear some structural relationship to arginine. They have been studied rather extensively during the period of this report.

2. The inhibitory effect shown by isopropyl biguanide is equally marked when the compound is administered in the yolk sac or the allantoic sac. As the virus is injected into the allantoic sac in both cases this suggests that no direct contact between the virus and the compound in the allantoic sac is necessary in order for inhibition to be demonstrated.

3. In the case of isopropyl biguanide 10.0 mg. per egg, which is about the maximum tolerated dose, produced the most marked inhibition of the virus. However, a significant inhibition is noted with a dose as low as 2.5 mg. per egg. In the case of benzoyl guanylurea 25.0 mg. per egg produced the maximum degree of inhibition, whereas 12.5 mg. per egg was ineffective. This compound was employed in the form of a suspension because of its low water solubility; hence it is not possible to make direct comparisons between the two compounds for activity on a weight basis.

4. In the case of isopropyl biguanide the inhibitory effect of a single dose of 10 mg. is still evident after the treated eggs have been incubated for three days at 35°C. Even after four days of incubation a slight effect is still apparent.

5. The inhibitory effect of isopropyl biguanide is definitely influenced by the dose of virus administered. With doses of about ten to one hundred ID_{50} the development of virus hemagglutinin is very markedly suppressed. With a dose of ten thousand ID_{50} slight inhibition is noted.

6. Isopropyl biguanide may be administered as long as twenty four hours after the infecting virus and still produce a marked suppression of virus hemagglutinin. Even after thirty-six hours a noticeable inhibition is found. This observation seems to indicate that the inhibitory effect is not due to interference with adsorption of the virus by the cells.

7. In the case of benzoyl guanylurea the inhibition of the virus is equally marked if the compound is given an hour before the virus or two hours after the virus. If injection of the compound is delayed for twenty-four hours, however, the effect is slight.

8. Isopropyl biguanide at a concentration of 2 mg. per ml. has no effect upon the hemagglutinating activity of the Lee influenza virus after exposures as long as twenty-four hours at 35°C. It does, however, under these conditions cause a more rapid loss of infectivity than is noted in a control preparation containing only the virus. This effect upon infectivity is not observed within twenty-four hours at 10°C. or in two hours at 35°C. It seems unlikely that it can entirely account for the inhibitory activity of the compound in the development of the virus.

9. Isopropyl biguanide has been shown to inhibit the development of Lee influenza virus in the chick embryo using infectivity as a measure of virus concentration, as well as the hemagglutinin measurement. Eggs treated with the compound have shown a virus titer at least one log unit lower than untreated eggs.

10. Experiments carried out in vitro have failed to show that isopropyl biguanide has any influence upon the adsorption of the Lee influenza virus by the chorio-allantoic membrane.

11. The effect of isopropyl biguanide on the growth of three other viruses in the chick embryo has been determined. In the case of PR8 influenza virus a slight but significant degree of inhibition was produced by a dose of 10 mg. of the compound per embryo. This is in contrast to the marked degree of inhibition produced in the case of the Lee virus.

12. Slight to moderate inhibition of the mumps virus in the chick embryo was noted when 10 mg. of isopropyl biguanide was administered in the allantoic sac, but only when the compound was injected the day following the virus inoculation. If administered an hour before the virus, or if half the dose was given at that time and half 48 hours later, no inhibition of virus hemagglutinin was observed.

13. Isopropyl biguanide did not delay the death of chick embryos infected with the virus of equine encephalomyelitis as compared with untreated control embryos.

14. Tissue culture studies have shown that both canavanine and isopropyl biguanide inhibit the growth of Lee influenza virus in concentrations only about one tenth as great as those required in the intact chick embryo. Tissue culture concentrations of 0.1 to 0.2 mg. per ml. are adequate for inhibition of the virus.

15. In tissue cultures, as in the intact chick embryo, an inverse relationship was shown between the degree of inhibition produced by isopropyl biguanide and the concentration of virus in the inoculum. Increasing concentrations of virus resulted in decreasing degrees of inhibition.

16. Attempts to interfere with the virus inhibition produced by isopropyl biguanide in tissue culture by means of various known metabolites involved in nucleic acid or protein metabolism have thus far given negative results. Among the metabolites tried have been arginine, lysine, adenine, p-aminobenzoic acid, folic acid, and desoxyribonucleic acid.

17. The stimulating effect of DL-citrulline on the development of Lee influenza virus hemagglutinin in the chick embryo has not been observed in tissue culture containing this amino acid.

18. A considerable amount of effort has been devoted to improving the extraction and purification of canavanine from jack bean meal. Ion exchange resins have been tried but a successful method has

not been achieved. Altogether some 35 grams of canavanine have been prepared by the original laborious process.

19. Additional compounds which have been prepared by synthesis or isolation include desaminocanavanine, canaline, cyanuric acid, 5-aminopyrimidinedione - 2,4; 5-nitropyrimidinedione - 2,4; 2-thio-4- oxypyrimidine; 5-α,α-dichloroacetamidopyrimidinedione - 2,4; and 2-methylmercapto-4-hydroxypyrimidine.

20. Considerable progress has been made toward the synthesis of compounds related to canavanine. Two compounds have been prepared in each of which the guanidino group has been linked to a carbon chain by means of an oxygen atom. This is the structure which is found naturally in canavanine. The two compounds which have been synthesized are carboxymethoxy guanidine and ethoxy guanidine sulfate. Having reached this point, the next step being attempted is the preparation of a similar compound containing an α-amino group which will represent the next lower homologue of canavanine.

PLANS FOR FUTURE WORK:

A. Immediate Future.

1. To complete the study of inhibition of Lee influenza virus by isopropyl biguanide in tissue culture, with the particular purpose of determining whether this inhibition can be reversed by any of the normal metabolites thought to be involved in nucleoprotein metabolism, such as adenine, uracil, nucleic acids, or amino acids. (now in progress)

2. To complete the study of the inhibition of Lee influenza virus by canavanine in the chick embryo. This work has been delayed by the limited availability of the compound. Sufficient has now been isolated from jack bean meal and purified to complete this work, and all that is now required is the availability of personnel and time.

3. To study the possible influence of canavanine on experimental infection in mice with Lee influenza virus.

4. To attempt to throw more light on the mechanism of the inhibition of the Lee virus by canavanine by employing the tissue culture method and investigating the possibility of reversing the inhibition by means of certain normal metabolites.

5. To examine the possible effect of canavanine on the growth of other viruses.

B. Long Range.

1. Preliminary tests have shown recently that cyanuric acid also acts as an inhibitor of Lee influenza virus in the chick embryo. This observation deserves as much study as we have devoted or plan